SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the chemopreventive effects of tyrosine kinase inhibitors in experimental animal models

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF NEUROSCIENCE

DEBRECEN, 2019

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The PhD Defense takes place at the Lecture Hall of the Institute of Pathology, Faculty of Medicine, University of Debrecen
1:00 p.m. 28th of May, 2019

1. INTRODUCTION

Protein kinases (PKs) play pivotal roles in cellular processes such as metabolism, proliferation, apoptosis, immune response, or nervous system functions. PKs regulate enzyme activity by phosphorylating cellular proteins and their dysregulation may lead to pathological conditions, i.e., different types of cancers or inflammatory diseases.

Malignant tumours are complex diseases, characterized by uncontrolled cell proliferation. To date, they have 8 main features: continuous proliferation, avoiding growth regulators, resistance to programmed cell death, replicative immortality, hiding from immune system, invasion, metastases, reprogramming metabolism. Protein kinases are essential elements in these signaling pathways. Therefore, PKs have become one of the most extensively investigated drug targets in the past two decades.

Kanner et al. in 1991 proposed that p120 is a substrate of both nonreceptor- and ligand-activated transmembrane receptor tyrosine kinases and of serine/threonine kinases (Ser/ThrK) and is a component of both mitogen-stimulated and tyrosine kinase oncogene-induced signaling pathways. This theory was proven and the "age of protein kinases" had begun.

To date, the human PK gene family consists of 518 members and can be categorized into nine groups. Among them, tyrosine kinases (TKs)—and their inhibitor molecules—are the most promising targets of cancer studies. TKs are classified as receptor and nonreceptor tyrosine kinases. Receptor tyrosine kinases (RTKs) are transmembrane proteins consisting of an extracellular ligand-binding domain and an intracellular kinase domain. Nonreceptor tyrosine kinases can be found in the cytosol and nucleus or in the inner part of the plasma membrane, participating in the regulation of cell proliferation or differentiation. The activation of TKs is under tight control. Their kinase activity is low in nonproliferating cells. On the contrary, TK expression is extremely increased in cancer cells, caused by ligand or receptor overexpression by various mechanisms. Therefore, reducing secondary signaling pathway activity with PTK inhibitors (PTKi) is expected to be effective in antitumour therapy. Moreover, with reducing the intensity of secondary molecular pathways, we could prevent malignant diseases in people exposed to severe carcinogen damage (e.g. people working with toxic material in chemical industry). We carried out our research from this point of view.

Compounds with PTKi activity from a range of plant secondary metabolites were highlighted by Hollósy and Kéri. Structure activity analysis and optimization with chemical modification followed by in vitro testing resulted in the development of several anticancer drug candidates.

DMBA is a widely used polycyclic aromatic hydrocarbon chemical carcinogen that initiates chemical carcinogenesis by inducing various oncogenic mutations resulting in lung tumour, squamous cell carcinoma, and vascular tumours (hemangiomas), as well as intestinal, mammary, uterine, or

hematologic tumours. Elevated expression of *Hras* and *Trp53* key onco/suppressor genes after treatment with the chemical carcinogen 7,12- dimethylbenz[a] anthracene (DMBA) has been previously observed. Our research group has developed an animal model for the investigation of alterations in onco/suppressor gene expression due to external carcinogenic agents, and this model had already been used to evaluate the carcinogenic effects of cytostatic drugs in humans, an analysis of the effects of ethylene oxide exposure seemed to offer further information on the usefulness of gene expression as a biomarker. DMBA is used in "short-term" in vivo experiments to determine possible chemopreventive effects of potential anticarcinogen compounds, which, if effective, should abrogate DMBA-induced expression. A number of chemopreventive drugs have already been investigated in this test system such as chalcone analogues as intermediary compounds of the flavonoid biosynthetic pathway or 2-Mercaptoimidazole derivative Afobazole.

In the first part of our experiments, we examined the potential antineoplastic and chemopreventive properties of four PTKi molecules. (1Z)-1-(3,4-dihydroxybenzylidene)-3,4- dihydro[1,4]oxazino[3,4-b]quinazolon-6(1H)-one (compound 1), N-(3-bromophenyl)-6,7-dimethoxy quinazolin-4-amine (compound 2), 2-benzyl-1-(4- hydroxyphenyl)-3-methyl-2,3-dihydroimidazo[5,1- b]quinazolin-9(1H)-one (compound 3) and 2-[(2E)- 2-(3,4-dihydroxybenzylidene)hydrazino]-N-(3- nitrophenyl)-2-oxoacetamide (compound 4).

In the second part of our study we assessed the short-term tissue-specific effects of imatinib mesylate on the expression of *Hras, Kras,* and *Myc* and *Trp53* genes in the bone marrow, brain, kidney, liver, lung, lymph nodes, spleen, and thymus of DMBA-treated mice.

2. AIMS

1. Investigation of the effect of the 4 compounds on the expression level of *Hras* gene in the liver, lungs, bone marrow and kidney of the DMBA-treated mice.
2. Demonstration of the effect of the 4 compounds on the expression level of *Trp53* gene in the liver, lungs, bone marrow and kidney of the DMBA-treated mice.
3. Assessment of the effects of imatinib mesylate on the expression of *Hras, Kras, Myc, Trp53* genes in the bone marrow, brain, liver, lungs, lymph nodes, spleen and thymus of the DMBA-treated mice.
4. Evaluating potential gene-gene interactions in connection with *Hras, Kras, Myc, Trp53* genes during tumourgenesis.

3. MATERIALS AND METHODS

All of the protocols used in the present study were approved by the Animal Experiment Committee of University of Pécs (BA 02/2000-16/2011). The mice were housed six animals per cage at an ambient temperature under a 12h:12h light:dark cycle with *ad libitum* access to chow food and water.

3.1. Effect of treatment with the 4 experimental compounds on the expression of *Hras* and *Trp53* genes in the liver, lungs, bone marrow and kidneys of DMBA-treated mice

3.1.1. Animals and treatment protocol

Six- to eight-week-old (20±4 g) conventionally maintained, CBA/Ca inbred H-2K haplotype mice (6 females in each group) were used for this experiment. Four experimental sets were created for each of the four experimental agents (Fig. 1). Two control (one negative and one positive) sets were established to compare the experimental results. The control and experimental mice were autopsied twenty-four hours after the last DMBA or experimental agent treatment. Compound 1, 2, 3 and 4 were supplied by Vichem Chemie Ltd., Budapest, Hungary.

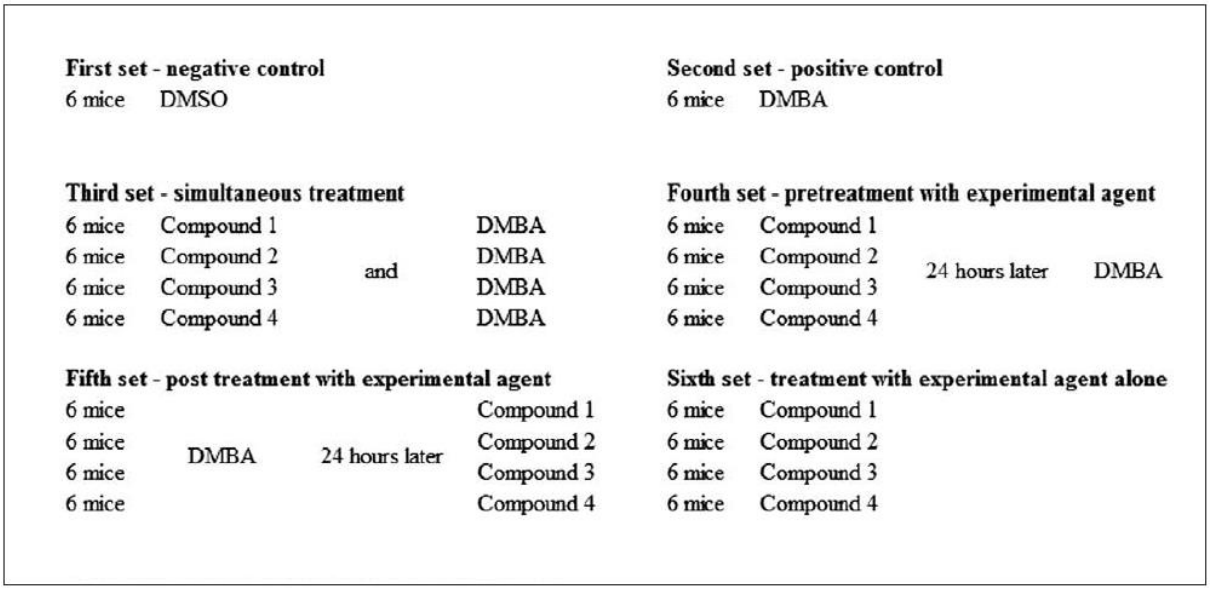

Figure 1. Experimental design of the first part of the study. DMSO: 10 mg/kg i.p., DMBA: 20 mg/kg i.p., compound 1-4: 10 mg/kg i.p.

3.1.2. RNA extraction and examination of gene expression

The liver, the lungs, the bone marrow and the kidneys of the animals were removed and 100 mg samples of each tissue from the respective groups pooled. After homogenization of the organs, total cellular RNA was isolated using TRIZOL reagent (Invitrogen, Paisley, UK). The RNA quality was assessed by denaturing gel-electrophoresis, and absorption measurement at 260/280 nm (A260/A280 was >1.8). After necessary dilution, 10 µg RNA was dot-blotted onto Hybond N+ nitrocellulose membrane (ECL kit, Amersham, Little Chalfont, UK) and hybridized with chemiluminescent specific probes for p53 and Ha-ras genes (Professor J. Szeberényi, University of Pécs, Hungary). Isolation of

RNA, hybridization and detection were performed according to the manufacturers' instructions. Chemiluminescent signals were detected on X-ray films, the films were scanned and evaluated by computer software Quantiscan (Biosoft, Cambridge, UK). The membranes rehybridized with constitutively expressed β-actin gene served as basis for comparison of gene expression. The results indicate the gene expression as a proportion of β-actin expression.

3.1.3. Statistical analysis

Statistical analysis was carried out with t-test implemented in SPSS statistics. Bars over the columns represent 2x standard deviation. The significance of the ratios was computed from the t-scores, and a threshold of $p<0.05$ was applied to define differentially expressed genes.

3.2. Evaluation of the potential chemopreventive effect of imatinib mesylate in DMBA-treated mice

3.2.1. Animals and experimental protocol

Six- to eight-week-old (25±5 g) conventionally raised NMRI inbred mice (n=12, 6 males and 6 females in each group) were involved in our study. Three experimental sets were created for the experimental agents (Fig.2). The first set of animals was treated intraperitoneally (i.p.) with vehicle (corn oil) and served as a negative control group. The second set of mice (positive control) was treated i.p. with a 20 mg/kg dose of DMBA dissolved in corn oil (both compounds were purchased from Sigma Aldrich, Budapest, Hungary). In the third group (experimental set), animals were simultaneously treated i.p. with 10 mg/kg imatinib mesylate (4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate, Novartis Pharma GmbH product (Gleevec, Glivec), and 20 mg/kg DMBA dissolved in corn oil. Mice were sacrificed 24 hours after the injections, and organs (liver, spleen, kidney, lung, thymus, lymph node, bone marrow, and brain) were harvested and snap-frozen in liquid nitrogen and then stored at - 80°C for further use.

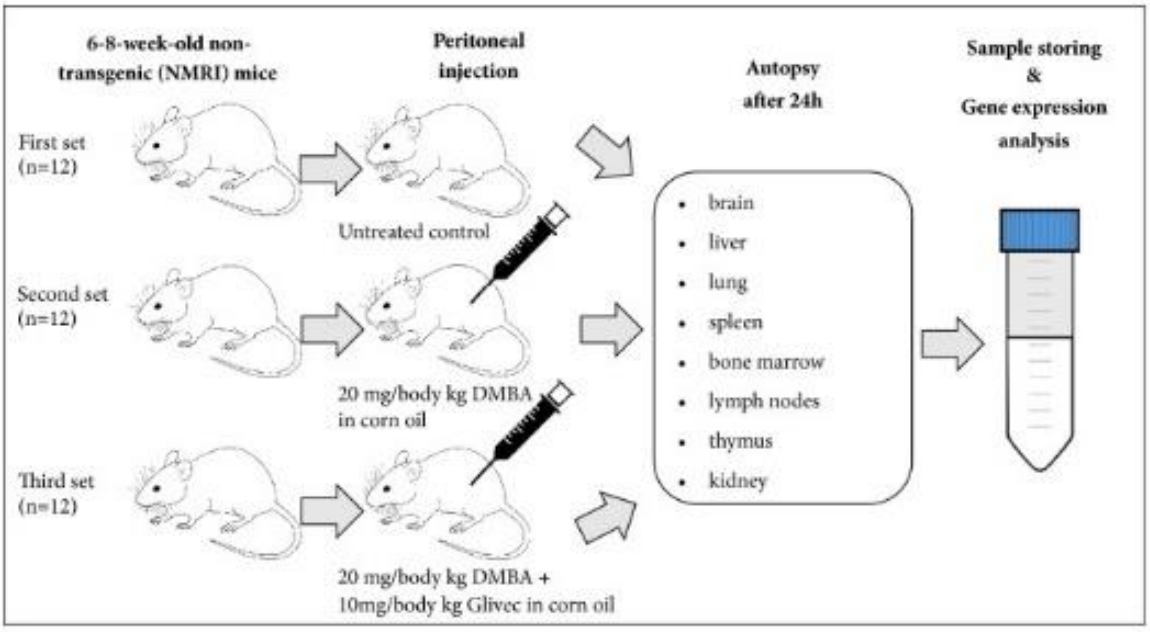

Figure 2. Experimental design of the second part of our study.

3.2.2. RNA extraction

100 mg tissue samples of each organ from the respective groups were homogenized in MagNA Lyzer Green Beads tubes (Roche (Hungary) Ltd.) using the MagNA Lyzer instrument (Roche (Hungary) Ltd.). Total RNA was isolated from the tissue lysates using the EXTRAzol RNA extraction kit (Invitrogen Life Technologies Magyarország Kft). The RNA quality was assessed by absorption measurement at 260/280 nm (A260/A280 was >1.8).

3.2.3. Gene expression investigation

One-step PCR including reverse transcription and target amplification was performed using Kapa SYBR FAST One-step RTqPCR Kit (Kapa Biosystems) on a LightCycler 480 qPCR platform with a 96-well format.

The specific primers (IDT) for mouse tumour suppressor genes (*Hras*, 5′-AATTGGGGGAGCAAGGACAT-3′); (*Kras*, 5′-TATCCTGCTTCCCATCAGTGTTC-3′); (*Myc*, 5′-GTTGTGCTGGTGAGTGGAGA-3′); (*Trp53*, 5′-CTTCACTTGGGCCTTCAAAA-3′) and for a housekeeping gene (*Gapdh*, 5′-CACATTGGGGGTAGGAACAC-3′) were used in the quantitative amplification.

RT-qPCR was initiated by 5 min. and 3 min. incubations at 42°C and 95°C, respectively, followed by 50 cycles (95°C for 10 s, 55°C for 20 s, and 72°C for 20 s) with a fluorescent reading taken at the end of each cycle. Each run was completed with a melting curve analysis (95°C for 5 s, 65°C for 60 s, and 97°C ∞) to confirm the specificity of amplification. Fluorescent values were calculated following the ΔΔCp method on Exor 4 software (Roche (Hungary) Ltd.) and gene expressions are reflected as relative quantification results.

3.2.4. Data analysis

Statistical analyses were performed using R software (http://www.r-project.org) and SPSS 21.0 software (SPSS Inc., IL, USA). The differences in mRNA expression levels were calculated using a two-tailed Student's t-test and were considered to be significant when $p<0.05$. Gene-gene interaction networks to demonstrate the relationship between genes in different organs/experimental sets were generated by the GeneMania Cytoscape 3.4.0 application. Physical, coexpression, and gene-gene interactions were evaluated. Heat map was constructed using Gene-E version 3.0.204.

4. RESULTS

4.1. Effect of treatment with the 4 experimental compounds on the expression of *Hras* and *Trp53* genes in the liver, lung, bone marrow and kidney of DMBA-treated mice

DMBA, a pluripotent chemical carcinogen, increased the expression of *Hras* and *Trp53* genes in a remarkable manner, in our "short-term" animal experimental model. DMBA, given alone, elevated the expression of the examined key onco/suppressor genes in every organ in nearly every experimental set. DMSO, which served as negative control and was used as a solvent for DMBA and the experimental agents, applied alone had very little effect on the examined genes in each of the examined organs. Generally, administration of the experimental molecules 24 hours prior to, simultaneously with, and 24 hours after DMBA exposure characteristically reduced DMBA-induced overexpression of the examined genes.

4.1.1. Liver

All of the four compounds reduced DMBA induced overexpression of *Hras* in the liver. Compound 1 reduced *Hras* expression in the fifth set, administered after DMBA exposure. Pre-treatment with compound 2 and 3 (fourth set) significantly ($p<0.05$) reduced expression of *Hras* to the level of the negative control. Simultaneous DMBA and compound 4 administration decreased expression of *Hras* gene (third set). The *Trp53* gene expression of the liver was reduced by compound 1 and 4 in the third, fourth and fifth set. In case of compound 1 no significance was found. Compound 4, while simultaneously administered with DMBA, reduced *Trp53* mRNA expression significantly ($p<0.05$) to the level of negative control.

4.1.2. The lungs

All four tyrosine kinase inhibitors reduced DMBA-caused *Hras* gene expression to the level of the negative control. Comparing to the positive control, the decreasing is significant ($p<0.05$). Compound 2 was an effective inhibitor in the fourth set. Simultaneously administered with DMBA (third set) compound 4 significantly ($p<0.05$) reduced *Hras* expression to the level of the negative control. DMBA-elevated *Trp53* mRNA level was significantly reduced ($p<0.05$) by compound 1 in the third, fourth and fifth set. Compound 2 and 3 treatment reduced elevated *p53* expression level (third, fourth, fifth set), the reduction is significant ($p<0.05$) in the fourth and fifth set.

4.1.3. Bone marrow

The DMBA-elevated *Hras* mRNA expression was reduced by the experimental compounds. Compound 2, 3 and 4 significantly reduced *Hras* expression ($p< 0.05$) in the third, fifth and sixth set. Compound 1 significantly ($p<0.05$) reduced *Hras* gene expression when the animals were pre or post treated with it (fourth and fifth set). All of the four compounds reduced the expression of *Trp53* gene in all the three experimental sets (third, fourth and fifth). Compound 1 and compound 3 decreased

expression of *p53* gene to the level of the negative control. Compound 4 significantly ($p<0.05$) reduced

Trp53 expression in all of the three treatment sets (third, fourth and fifth). The downregulation by compound 2 was not significant in the third set, but in the pre and post-treatment sets (fourth and fifth) *Trp53* mRNA level decreased in the bone marrow.

4.1.4. Kidneys

All four compounds reduced *Hras* gene expression comparing to the positive control. Compound 1, 2 and 3 significantly reduced *Hras* expression in the third, fourth and fifth set ($p<0.05$). Compund 1 and compound 2 reduced *Hras* mRNA expression to the level of the negative control. Comparing to the third and fourth sets, compound 3 showed greater *Hras* reduction in the fifth set. The expression of *Trp53* tumour suppressor gene was found to be higher in the kidneys than in other examined organs. DMBA treatment (positive control) did not increased *Trp53* expression in the kidneys. Compound 1 and compound 2 reduced *Hras* and *Trp53* gene expression in all of the experimental settings where they were administered. The reduction is significant ($p<0.05$), except for *p53* in the fourth set of compound 2. Compounds 3 and 4 reduced *Hras* expression significantly ($p<0.05$) in the third, fourth and fifth set. Compound 3 administered before and after DMBA exposure increased *Trp53* gene expression in the kidneys (third and fourth set). Compound 4 administered with DMBA and alone increased *Trp53* gene expression in the kidneys.

4.2. Evaluation of the potential chemopreventive effect of imatinib mesylate in DMBA-treated mice

4.2.1. Gene epression

Bone Marrow

In the bone marrow, DMBA injection decreased the expressions of *Hras, Kras,* and *Myc,* respectively, and increased *Trp53* expression. DMBA+imatinib mesylate administration further decreased the *Hras, Kras,* and *Myc* expressions. Compared to the negative control, significantly lower *Kras* expressions were found in the second *($p<0.05$)* and third sets of mice *($p<0.05$).* The combined treatment also decreased the expression of the tumour suppressor *Trp53* to a significant extent (*$p<0.05$*), first (control) versus third (DMBA + imatinib mesylate) set.

Brain

Compared to the negative controls, DMBA administration resulted in increased gene expressions in the brain; however, these changes were found to be nonsignificant. Combined administration of

DMBA and imatinib mesylate decreased the expressions of the studied genes; however, these alterations were not significant either.

Kidney

DMBA increased the expressions of the *Hras, Kras,* and *Myc,* respectively, and the expression of the *Trp53,* as well. The simultaneous administration of DMBA and TKI reduced the expression of all the investigated genes.

Liver

In the liver, DMBA administration lowered the expressions of *Hras, Kras, Myc,* and *Trp53,* respectively. As a result of the combined DMBA+TKI administration, the decrease in the expression of these genes became reduced.

Lung

In the lung, mRNA expressions of the *Kras* ($p<0.05$), *Myc,* and *Trp53* genes were increased, while the *Hras* expression was decreased following the DMBA injection. Simultaneous treatment with DMBA and TKI led to decreased the expression of protooncogenes (*Hras, Kras,* and *Myc*) and increased *Trp53* mRNA levels.

Lymph Nodes

In the lymphoid tissues, DMBA decreased the *Hras* expression and increased the *Kras* and *Trp53* expressions, that remained unchanged after the combined administration with DMBA+TKI. However, the expression of *Myc* was increased by DMBA and decreased as a result of DMBA+TKI combination. However, this change in mRNA expression was not statistically significant.

Spleen

Hras and *Kras* gene expressions were decreased after DMBA injection, although they did not change after DMBA+TKI administration. In turn, DMBA induced increased expressions of *Myc* ($p<0.05$) and decreased *Trp53* expressions after treatment (DMBA+TKI).

Thymus

In the thymus, DMBA increased the expressions of *Kras, Myc* and *Trp53,* respectively, while decreasing the *Hras* expression. As a result of combined administration of DMBA+imatinib mesylate, the expressions of *Kras* and *Trp53* were found to be reduced compared to the negative control. Additionally, the expression of *Myc* showed an increase, while the expression of *Hras* remained unaltered after the combined injections.

4.2.2. Gene network

We observed significant alterations in gene expressions in the bone marrow, lung, and spleen. Our network analysis revealed that *Hras, Kras,* and *Myc* protooncogenes and*Trp53* tumour suppressor gene have extensive connections to other regulatory genes. *Zhx2* (also known as*RAF*) is a homodimeric transcription factor that belongs to the zinc fingers and homeoboxes gene family, *Abi1* (abl interactor 1) is an adaptor protein that facilitates several signal transduction pathways, regulates actin polymerization and cytoskeleton remodeling, and therefore has a role in cell proliferation. *Tcf4* (transcription factor 4) is essential for neuronal development, and *Tsc2* (TSC complex subunit 2) gene codes a tumour suppressor protein (tuberin), mutation of which (together with mutation of hamartin, coded by *Tsc1*) causes tuberous sclerosis complex. *Huwe1* encodes an E3 ubiquitin ligase protein that is responsible for ubiquitination and degradation of the antiapoptotic protein MCL1 (myeloid cell leukemia sequence 1 (*Bcl2*-related)). *Cdkn2a* (cyclin dependent kinase inhibitor 2a) is an important tumour suppressor gene, having at least three alternative spliced variants that code two CDK4 inhibitors and one p53 stabilizer protein, therefore playing a pivotal role in cell cycle G1 control. *Nde1* (nudE neurodevelopment protein 1) gene codes a protein that has essential role in microtubule organization, mitosis, and neuronal migration, mutation of which can be associated with lissencephaly. *Kmt5a* (lysine methyltransferase 5a) codes a protein that is a transcriptional repressor and is important for cell proliferation and chromatin condensation. *Mcm4* (minichromosome maintenance complex component 4) gene codes a protein that is highly conserved and important for initiation of eukaryotic genome replication. *Eif4e* (eukaryotic translation initiation factor 4E) functions as a protooncogene; its product helps the initiation of translation.

5. DISCUSSION

5.1. Effect of treatment with the 4 experimental compounds on the expression of Hras and Trp53 genes in the liver, lungs, bone marrow and kidneys of DMBA-treated mice

As we use the expression of *Hras* onco- and *Trp53* tumoursuppressor genes as an early biomarker of chemical carcinogenesis, the increase of their expression shows the carcinogen exposure of the tissues. DMBA increased the expression of the examined genes in the examined organs. However DMBA did not exert remarkable effect of *Trp53* gene expression in the kidneys, due to lipophilic distribution and also the need for its metabolic activation. DMBA is a carcinogen foremost in the lungs (CYP1A1 highly expressed), liver (CYP1A2 highly expressed) and in other organs which highly express activating enzymes.

Compounds 1, 2 and 3 given alone in all of the experimental settings, in all of the examined organs exerted less expression increasing effect, than the DMBA treated group, indicating chemopreventive effect of the examined quinazolin type molecules.

Compound 1 and compound 2 reduced both DMBA increased *Hras* and *Trp53* gene expressions in all of the examined organs in all of the experimental settings due to PTKi effect. The inhibitory effect of certain PTKi agents lasts for at least 48 hours on PTK secondary signal transduction pathway. This underpins our results in the applied time period, 24 hours prior to, simultaneously with and 24 hours after DMBA exposure.

A reduction in *Hras* expression corresponds to inhibition of Ras Extracellular signal-Regulated Kinase (ERK) pathway (which directs signals to the immediate-early genes e.g. cfos, c-jun up-regulating transcriptional factors, enhancing cell proliferation), thus exerting a chemopreventive effect. Moreover Liu et al. found with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay-based flow cytometry that PTK inhibition reduced the protein level of cyclin D1, a cell cycle-related protein. This reduced the activation of the ERK pathway and also the inducibility of cyclin D1, leading to lower cell proliferation.

The same is true for compound 3 and 4, except for the kidneys, where only *Hras* gene expression moderately decreased. This may be explained by the accumulation of the lipophilic agents in the kidneys and the mutual significance of *Trp53* overexpression in malignant diseases: early *Trp53* overexpression is partially a consequence of *Hras* (and of c-myc as well) overexpression caused by carcinogen exposure. *Trp53* overexpression is a crucial component in the initiation of EGFR TK pathway, resulting in reduction of cell proliferation via G0G1 cell cycle arrest and induction of apoptosis, with dramatic clinical relevance e.g. in patients with non-small cell lung cancer (NSCLC).

Compound 4 treated group – alone and in coadministration with DMBA as well – elevated *Trp53* gene expression in the kidneys in each setting. This result differs from expected based upon previous in

vitro experiments. Since gene expression patterns of compound 1, 2 and 3 treatment confirms the in vitro experiments, further investigations of the effect of compound 4 on the kidneys are needed. Since p53 has a Janus-faced function it is pro-apoptotic and also transactivates genes whose products act in an anti-apoptotic manner. Our test system is able to indicate other apoptotic pathways, in order to determine the events behind compound 4 elevating *Trp53* expression, other apoptotic genes should also be investigated in future experiments.

Activation of receptor tyrosine kinase signaling, including EGFR, has been implicated in the development of malignant disease e.g. high-grade gliomas and hemangioblastomas. Moreover, Chang et al. revealed in patients with primary lung cancers (with corresponding lymph node metastases), that p53 and EGFR mutations usually precede lymph node metastasis (but heterogeneity of EGFR expression should be considered in therapeutic aspects). Thus, the complex nature of EGFR biology allows potential opportunities for EGFR inhibitors in a number of areas of cancer therapy, including proliferative, angiogenic, invasive and metastatic aspects. For example, the alkaloid staurosporine acts as a potent inhibitor of protein kinase C and PDGF receptor proteins. Molecules blocking growth factor signaling at several points by inhibiting protein kinase C, phosphoinositide specific phospholipase C and inositol(1,4,5) trisphosphate induced Ca2+ release supports in vivo antitumour mechanisms and enhance beneficial health effects of chemopreventive agents. Inhibition of the mentioned signaling pathways may explain the effects of these experimental agents and corresponds with a great concordance to the results of the in vivo experiment detailed in this paper. In our previous studies with the in vivo model we found that the reducing effect of the investigated compound also depends on the administration schedule. A chemopreventive agent might interact in several ways with the multistage carcinogenesis for example by modifying transmembrane transport, modulating metabolism, blocking reactive species, inhibiting cell replication, maintaining DNA structure, modulating DNA metabolism and repair, and controlling gene expression. For example a blocking agent inhibiting the activation of a carcinogen to its ultimate carcinogenic form is more potent given prior to or with the carcinogen itself. On the other hand a blocking agent that modifies metabolising enzyme systems acts later. To evaluate whether the examined compounds have another effect besides kinase inhibition, or have a time-dependent effect on DMBA carcinogenesis, we established three experimental sets depending on the order of the administered DMBA and experimental compounds. In recent experiment some tendencies may be found that might help further investigations about potential chemopreventive mechanism of investigated compounds.

5.2. Evaluation of the potential chemopreventive effect of imatinib mesylate in DMBA-treated mice

Major results of the present study include that short-term DMBA treatment (i) elevated the expression of all the three protooncogenes (*Hras, Kras,* and *Myc*) in the brain and kidneys; (ii) increased the level of*Kras* and *Myc* in the lung, lymph nodes and thymus; (iii) increased the expression of the tumour

suppressor gene *Trp53* that can be considered an adaptive physiologic countermeasure in response to a chemical carcinogen.

In the bone marrow and liver, DMBA decreased the expression level of *Hras, Kras,* and *Myc*. This observation might be explained by the fact that DMBA is a carcinogenesis inducer, and it is usually applied simultaneously with a carcinogenesis promoter, e.g., 12-O-tetradecanoylphorbol 13-acetate (TPA). Therefore, in case of the bone marrow and liver, DMBA might not be enough for complete tumourigenesis. In the spleen, the elevated level of *Myc* was the only prominent and significant alteration in the gene expression pattern. Several studies have elucidated the role of *Myc* in tumourigenesis. Probably the best-established association is that nearly every case of Burkitt's lymphoma involves rearrangement and therefore overexpression of *Myc* with a regulatory element of immunoglobulin heavy or light chains or other nonrandom somatic mutations of the gene. The results of the aforementioned studies correlate with our findings.

The increased expression of the examined four genes gain more importance in the context of their extensive gene network. *Zhx2* (also known as *RAF*) has previously been associated with Hodgkin lymphoma and hepatocellular carcinoma; *Abi1* (abl interactor 1) has a role in colorectal carcinoma development and invasion and also in neuroblastoma propagation. Aberrant function of *Tcf4* (transcription factor 4) has been reported in glioblastoma and in colorectal tumours. *Tsc2* (TSC complex subunit 2) gene codes a tumour suppressor protein (tuberin), mutation of which have been associated with tumours in the brain, lungs, kidneys, skin, heart, uterus, and eyes. *Huwe1* encodes an E3 ubiquitin ligase protein that is required for the development of colorectal carcinoma and ovarian tumours. *Cdkn2a* (cyclin dependent kinase inhibitor 2a) is an important tumour suppressor gene predisposing to several tumours, e.g., urothelial carcinoma, hereditary melanoma, pancreas cancer, or non-small-cell lung cancer. *Nde1* (nudE neurodevelopment protein 1) gene codes a protein that has essential role in microtubule organization and mitosis, and recent studies have elucidated its potential role in acute or chronic myeloid leukaemia. *Mcm4* (minichromosome maintenance complex component 4) has been reported to be upregulated in ovarian cancer, skin cancer, or esophageal carcinoma. *Eif4e* (eukaryotic translation initiation factor 4E) functions as a protooncogene; its product has been suggested to regulate expression of proteins that are crucial for cell cycle progression, cell survival, and motility. A growing body of evidence implicates this translational factor in cell transformation, tumourigenesis, or tumour progression, e.g., in case of prostate cancer, lymphomas, CML, or lung cancers.

This network and series of events offers numerous opportunities to effectively influence the process of tumourigenesis.

In the lungs, the expression of protooncogenes (*Hras, Kras,* and *Myc*) and their connections to other genes coding transcription factors or cell proliferation regulators (e.g., *Tcf4, Abi1,* and *Zhx2)*

prominently decreased as a result of the short-term combined DMBA+TKI treatment, while the expression of *Trp53* gene increased. Comparing to the negative control, the decrease in *Kras* expression was significant. In the bone marrow, DMBA+TKI combined treatment significantly decreased the expression and gene interactions of the *Kras* and *Trp53*. DMBA+TKI treatment could significantly decrease the DMBA-induced increase in the expression and gene interactions of *Myc* protooncogene. The expression of the tumour suppressor *Trp53* also decreased following the combined treatment; however, this decrease was not significant.

Outcomes of our short-term experiment suggest that protein tyrosine kinase inhibitor treatment (imatinib mesylate) simultaneously administered with the chemical carcinogen, DMBA, might have an impact on the expression pattern of the examined protooncogenes (*Hras, Kras,* and *Myc)* and tumour suppressor gene (*Trp53*), therefore on the tumourigenesis, controlled by these genes.

Imatinib mesylate was able to decrease significantly the expression of *Kras* oncogene in the bone marrow and in the lung, as well as the expression of *Myc* oncogene in the spleen. Additionally, *Myc* mRNA expressions were tended to be lowered in the bone marrow, brain, kidneys, lungs, and lymph nodes and we also observed tendencies in the *Hras* mRNA expressions to be decreased in the bone marrow, kidneys, and lungs, although these changes were not statistically significant. The reduced expression of these oncogenes may be attributed to the kinase inhibitor effect of imatinib mesylate, as described by other recent studies. Among others, Lorri Puil et al. reported that BCR-Abl was able to activate Ras signaling in CML, by creating a direct link between Grb2 and mSos1 that are responsible for the conversion of inactive GDP-bound form of Ras into the active, GTP-bound form. Therefore, inhibiting BCR-Abl kinase activity may downregulate Ras signaling in CML. Besides Ras signaling, BCR-Abl kinase can indirectly activate *Myc* either through the Janus-activated kinase 2 (JAK2) pathway or by the mitogen-activated protein kinase (MAPK) pathway. It is tempting to speculate that imatinib might have decreased the expression of *Myc* well before its DMBA-induced overexpression.

Kras, Hras, and *Myc* are the executive elements of numerous oncogenic pathways, so they can be favorable to inhibit a common point of tumourigenesis by one molecule. p53 is the best characterized tumour suppressor protein, as it is able to induce cell cycle arrest or cell death in response to hypoxia and incorrigible genetic mutations, while mutations of *Trp53* gene have been associated with more that 50% of human tumours. There is growing evidence that these mutations are ‘loss-of-function' mutations; however, missense mutations may result in simultaneous gain of functions that have usually detrimental effect to the cell. Numerous studies have reported that mutant p53 played a key role in tumour development, progression, and invasion of several cancer types, e.g., in case of breast cancer, lung cancer, colorectal cancer, different brain tumours, and gastric adenocarcinoma. In our present study, short-term imatinib mesylate treatment administered simultaneously with DMBA resulted in a prominent increase in the *Trp53* expression in the lung, while decreasing it in all the other

tissues. These data indicate a possible ‘gain-of-function' mutation in the gene of the tumour suppressor p53 protein and that imatinib mesylate attempted to decrease the level of this aberrant protein.

Based on our recent and previous findings we suggest that imatinib mesylate is a promising chemotherapeutic agent for prevention and management of several malignant tumours by decreasing the mRNA expression of the protooncogenes and the mutant *Trp53* gene.

6. MAIN RESULTS AND CONCLUSIONS

1. Compound 1 and 2 – examined in the first part of our study – decreased the *Hras* and *Trp53* expression that was elevated by DMBA, in all examined organs, in all experimental sets, due to its PTKi effect.
2. We could demonstrate similar results in case of compound 3 and 4. The only difference is that they could only decrease the expression of *Hras*. However, these results suggest that these compounds might have chemopreventive effect.
3. In the second part of our study DMBA increased *Hras, Kras* and *Myc* gene expressions in most of the examined organs that is in correlation with the carcinogenic potential of the compound. It is also a very important finding that these examined genes have a widespread gene network, offering us numerous opportunities to influence tumourgenesis.
4. An outstanding result is that DMBA+TKI (imatinib mesylate) treatment was able to moderate the level of examined proto-oncogenes in many organs – e.g. in the lungs, bone marrow, kidneys – suggesting a potential chemopreventive effect of the drug.
5. Another result is that imatinib decreased the expression of *Trp53* – that probably went through a 'loss-of-function' mutation – which might have a DNA-protectant role in case of carcinogenic damages.

LIST OF PUBLICATIONS

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Registry number: DEENK/77/2019.PL
Subject: PhD Publikációs Lista

Candidate: Péter Gergely
Neptun ID: EIF9IY
Doctoral School: Doctoral School of Neurosciences
MTMT ID: 10036942

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Total IF of journals (all publications): 15,007
Total IF of journals (publications related to the dissertation): 3,333

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

20 March, 2019

DEBRECENI EGYETEM
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ACKNOWLEDGEMENTS

In memoriam Professor István Ember (1952-2013).

I am particularly grateful for the assistance given by Professor István Kiss, Dr. János Bencze, Dr. Ferenc Budán, Dr. Ágoston Ember, Dr. Katalin Fogarasi-Nuber, Dr. Peter Fülöp, Dr. Katalin Gombos, Dr. Krisztina Holló, Dr. András Huszár (†), Dr. János Iványi, Dr. Balázs Kádár, Dr. György Kéri(†), Dr. Andrea Kurucz, Dr. Géza Mezey, Dr. Balázs Murnyák, Dr. Árpád Németh, Dr. Zsuzsa Orsós, Dr. László Őrfi, Dr. István Szabó, Dr. Péter Török, Dr. Tímea Varjas, Ms. Zsuzsa Bayer, Ms. Jánosné Csidei, Ms. Tímea Csörge, Ms Tiborné Déri, Ms. Mária Fóris, Ms. Csabáné Harth, Ms. Mónika Herczeg, Mr. Norbert Nádasdi, Ms. Zsuzsanna Oláh, Ms. Erzsébet Pál, Ms. Krisztina Rónai, Mr. Péter Szabolcsi, Ms. Szántódiné Ágnes Molnár, Ms. Szilvia Varga and Ms. Mária Wenczler. Without their precious support it would not be possible to conduct this research.

I would like to express my sincere gratitude to my advisor Tibor Hortobagyi for the continuous support of my PhD study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

I would like to express my very great appreciation to Professor Miklós Antal and Professor János Kappelmayer for their valuable and constructive suggestions during the planning and development of this research work. Their willingness to give their time so generously has been very much appreciated. In particular, I am grateful to Dr. László Herczeg, my former boss for letting me do this research.

Last but not the least, I would like to thank my family: my helpmeet, Zita Szombathy, my daughters Nóri and Sári, my parents and to my brother for supporting me spiritually throughout writing this thesis and my life in general.